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Effects of Aging on the Neutral and Polar Lipid Composition of *Biomphalaria glabrata* under Laboratory Conditions

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Abstract. High performance thin layer chromatographic analysis was used to determine the effects of aging on the lipid composition of the pulmonate snail, *Biomphalaria glabrata.* Whole bodies of three size (age) classes were used for analysis: (A) 5–7 mm juvenile snails, (B) 9–10 mm immature adults, and (C) 16–18 mm sexually mature adults. There was no difference in free sterol concentrations among any of the size classes. The concentrations of free fatty acids and triacylglycerols were significantly increased in A and B compared to C. As snails aged, the triacylglycerol and free fatty acid fractions declined significantly. The results indicate that sexually mature snails contain less depot lipids in the form of triacylglycerols and free fatty acids than the immature snails. The concentrations of phosphatidylcholine and phosphatidylethanolamine were significantly increased in A and C compared to B. Shells of sexually mature snails contained free sterols, free fatty acids, and cholesteryl esters in quantifiable amounts. Egg masses, newly hatched snails (< 1 mm), and 2–4 mm juveniles with shells contained free sterols, free fatty acids, and triacylglycerols in quantifiable amounts.

INTRODUCTION

Previous studies (see review in Fried & Sherma, 1990) have examined lipids in *Biomphalaria glabrata* (Say, 1816) of mixed sizes (ages) maintained on various diets. The diets have consisted mainly of lettuce, lettuce plus Tetramin, hen's egg yolk plus Tetramin, and hen's egg yolk plus lettuce. In these studies, the major lipids found in the snails were triacylglycerols, free fatty acids, free sterols, and phosphatidylethanolamine.

We initiated the present work on the effects of snail aging on the lipid composition of *B. glabrata,* because most previous studies did not take into consideration the size (age) of the snails used for analysis. Our study reports on the analysis of the neutral lipids and polar lipids (phospholipids) in whole bodies of snails of known size (age) classes as described in the Materials and Methods. Additional lipid studies were also done on juvenile snails with intact shells. Since the results of lipid analysis of *B. glabrata* egg masses or shells are not available, our study also provides such information.

MATERIALS AND METHODS

Sixty juvenile *Biomphalaria glabrata* snails, about 5–8 mm in shell diameter, were obtained from Dr. Fred A. Lewis, Schistosomiasis Laboratory, Biomedical Research Institute (Rockville, Maryland, USA). Groups of 20 snails were maintained at 23 ± 1°C in aerated glass containers (mason jars) each containing 800 mL of artificial spring water (ASW) (Ulmer, 1970) under diffuse overhead fluorescent light for 12 hr per day. These cultures were fed *ad libitum* on boiled Romaine lettuce leaf. Food and water were changed twice weekly in all cultures. These snails were allowed to grow for another 8–12 weeks until they became sexually mature and oviposited in the cultures. Strips of plastic, 4 cm × 4 cm, were placed in the cultures to facilitate egg laying. The strips containing eggs were removed to finger bowl cultures (11 cm diameter with 150 mL ASW) containing 2 cm × 2 cm pieces of lettuce, and the juveniles were allowed to attain a size of 4–5 mm. Some juvenile snails from these cultures were used for lipid analysis while others were transferred to mason jar cultures to raise young adults and sexually mature adults.

Lipids were analyzed in the whole bodies of snails minus their shells for three size classes and were designated as A, B, and C. Class A consisted of 5–7 mm juvenile snails. Class B consisted of 9–10 mm sexually immature adults. Class C consisted of 16–18 mm sexually mature adults. Snail bodies were removed by gently crushing the shells with a hammer and removing the bodies with forceps under a dissecting scope. Individual samples were used for analysis of C, whereas pools of three and five snails were used for B and A, respectively. Snails belonging to the 0.7–1 mm newly hatched group and 2–4 mm young juveniles were not removed from their shells, and lipid analyses of these snails were done with the shells intact. A total of 21 egg masses containing 366 eggs were also used for quantitative lipid analysis.

Lipids were extracted from all samples and quantita-

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Table 1

Weight percent of neutral lipids and phospholipids in the whole bodies of snails of different size classes.*

Size class	Shell diameter	Age (wk)	Neutral lipids		
			Free sterols	Free fatty acids	Triacylglycerols
A++	5–7	8–9	0.0757 ± 0.0084	0.0306 ± 0.0059	0.278 ± 0.10[a]
B+	9–10	12–14	0.0429 ± 0.0068	0.0577 ± 0.0060	0.0821 ± 0.017
C	16–18	20	0.0547 ± 0.0065	0.00453 ± 0.0016	0.0211 ± 0.0037
			Phospholipids		
			Phosphatidylcholine	Phosphatidylethanolamine	
A++	5–7	8–9	0.817 ± 0.15[a]	0.126 ± 0.014	
B+	9–10	12–14	0.175 ± 0.049	0.0432 ± 0.016	
C	16–18	20	0.659 ± 0.077	0.141 ± 0.017	

* Snail bodies: Mean (weight %) ± standard error, n = 4 snails for each sample (unless indicated otherwise).
+ n = 4 with each sample pooled from 3 snails.
++ n = 4 with each sample pooled from 5 snails.
[a] n = 3.

tively analyzed as described by Schneck et al. (2003). Briefly, following chloroform-methanol (2:1) extraction, samples were treated with Folch wash (0.88% KCl), dried under N_2 gas in a warm water bath (approximately 40°C), and the residue was reconstituted in 100–200 μL of chloroform-methanol. Quantitative analysis for neutral lipids was performed by high performance thin layer chromatography (HPTLC) on Whatman (Clifton, New Jersey) LHPKDF silica gel plates with zone detection by use of phosphomolybdic acid (PMA) spray reagent. Quantitative analysis for polar lipids was performed by HPTLC on the same layers using 10% cupric sulfate spray reagent for detection. A neutral lipid standard (Non-Polar Lipid Mixture-B, Matreya, Inc., Pleasant Gap, Pennsylvania) containing equal amounts of cholesterol, oleic acid, triolein, methyl and cholesteryl oleate provided markers for free sterols, free fatty acids, triacylglycerols, methyl and cholesteryl esters, respectively. The standard for polar lipid analysis (Polar Lipid Mix, Matreya Inc.) containing equal amounts of cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) provided markers for free sterols, PE, and PC. Visible mode quantitative densitometric analysis was performed by use of a Camag (Wilmington, North Carolina) TLC Scanner II with CATS-3 software.

RESULTS AND DISCUSSION

The samples analyzed for neutral lipids showed zones with migration comparable to the standards at R_f values of 0.21 (cholesterol), 0.25 (oleic acid), 0.55 (triolein), and 0.67 (methyl oleate) in the petroleum ether-diethyl ether glacial acetic acid (80:20:1) mobile phase of Mangold (1969). The R_f values of cholesteryl oleate sample and standard zones were 0.62 in the hexane-petroleum ether-diethyl ether-glacial acetic acid (50:2:5:1) mobile phase of Smith et al. (1995). The cholesteryl oleate sample zone was only detectable in the sexually mature adult size class, and the body weight percentage was determined to be 0.0482 ± 0.0063% (n = 4). Samples analyzed for phospholipids showed sample and standard zones at R_f values of 0.33 for PC and 0.47 for PE in the chloroform-methanol-water mobile phase (65:25:4) of Wagner et al. (1961). The LPC in the polar lipid standard was not detected with the cupric sulfate reagent at the concentrations applied, and zones that might have been LPC were not seen in samples.

Table 1 presents neutral lipid and phospholipid percentages for whole snail bodies of the three size classes (A, B, and C). The data were analyzed using ANOVA single factor comparison with $P < 0.05$ being considered significant. There was no difference in free sterol concentrations between any of the size classes. The concentrations of free fatty acids and triacyglycerols were significantly increased in A and B compared to C. The concentrations of PC and PE were significantly increased in A and C compared to B.

The egg masses, newly hatched snails, and juveniles contained quantifiable levels of free sterols, free fatty acids, and triacylglycerols. The egg masses (n = 1) had concentrations of free sterols, free fatty acids, and triacylglycerols of 0.00632, 0.00209, and 0.00184 percent, respectively. Six newly hatched juveniles (< 1 mm, 1–2 days old) were pooled to obtain a single sample, analysis of which showed concentrations of free sterols, free fatty acids, and triacylglycerols of 0.0517, 0.0283, and 0.238 percent, respectively. Six juvenile snails (2–4 mm, 3–5 weeks old) were pooled to obtain a single sample. These juveniles were found to have concentrations of free sterols, free fatty acids, and triacylglycerols of 0.105, 0.0226, and 0.0915 percent, respectively.

The shells of four sexually mature adults (16–18 mm) were analyzed for neutral lipids. Prior to analysis, the shells were washed five times with ASW to remove any residual hemolymph or snail tissue associated with the shell. The quantifiable neutral lipids in the shells were free sterols, free fatty acids, and cholesteryl esters, which were found to be present at concentrations of 0.00227 ± 0.00094, 0.00751 ± 0.0029, and 0.000938 ± 0.00034, respectively. Lipid studies on gastropod shells appear not to be available, but CoBabe & Pratt (1995) have found that bivalve shells contain fatty acids, cholesterol, phytadienes, ketones, and alkanes. Further studies on lipids in gastropod shells are warranted.

The most dramatic change in neutral lipids as a function of snail aging occurred in the triacylglycerol and free fatty acid fractions. Sexually mature adults had 13× and 7× less concentrations of triacylglycerols and free fatty acids, respectively, than the juvenile snails. Triacylglycerols and free fatty acids are storage depots in snails (Fried, 2003), and presumably younger sexually immature snails contain more of these lipids in their tissues than do the sexually mature adults. Free sterols are important to the structural integrity of cells and comprise major components of cell membranes. As expected, free sterol concentrations remained constant during snail aging. Phospholipids also play an important role in the structural integrity of cells and constitute a major component of cell membranes. We cannot explain the differences in concentrations of phospholipids between the size classes.

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